The sterile insect technique and the Mediterranean fruit fly: assessing the utility of aromatherapy in large field enclosures

Todd E. Shelly[1,*], Donald O. McInnis[2] & Pedro Rendon[3]

[1]*USDA-APHIS, 41-650 Ahiki St., Waimanalo, HI 96795 and Center for Conservation Research and Training, University of Hawaii, Honolulu, 96822 USA;* [2]*USDA-ARS, 2727 Woodlawn Ave., Honolulu, HI 96822 USA;* [3]*USDA-APHIS, 4a Calle 12-62, Zona 10, Guatemala City, Guatemala*

Accepted: 17 May 2005

Key words: Ceratitis capitata, Diptera, Tephritidae, mating competitiveness, sterile male technique, ginger root oil, overflooding ratio, integrated pest management

Abstract

The sterile insect technique (SIT) is widely used in integrated programs against tephritid fruit flies, particularly the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae). Unfortunately, the mass-rearing procedures inherent to the SIT often lead to a reduction in the male mating competitiveness. One potential solution involves the prerelease exposure of males to particular attractants. In particular, male exposure to ginger root oil [*Zingiber officinale* Roscoe (Zingiberaceae); hereafter GRO], has been shown to increase mating success dramatically in field cage trials. To evaluate more rigorously the effectiveness of GRO exposure, we here describe two projects that compared levels of egg sterility or pupal yield, respectively, following the release of wild flies and either GRO-exposed (treated) sterile males or GRO-deprived (control) sterile males in large field enclosures. In both projects, sterile males from a genetic sexing strain were exposed as adults to GRO for 24 h while held in large storage boxes. In Hawaii, we dissected eggs from fruits to determine the percentage of egg hatch at four overflooding ratios, ranging from 5 : 1 to 60 : 1 (sterile : wild males), and found that, at all four ratios, the proportion of unhatched (sterile) eggs was significantly greater in enclosures containing GRO-exposed males than control males. In Guatemala, we allowed larvae to develop in fruits and counted the number of pupae produced. At the only overflooding ratio tested (25 : 1), pupal yield was approximately 25% lower for enclosures containing GRO-exposed males than control males, although this difference was not statistically significant. An explanation for the differing outcomes is proposed, and the implications of these findings for the SIT are discussed.

Introduction

The sterile insect technique (SIT) is an environmentally benign approach for suppressing or eradicating insect pests and is widely used in integrated programs against tephritid fruit flies, particularly the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) (Hendrichs et al., 1995, 2002). The technique involves mass production of males of the target species and release of irradiated (sterilized) males into the environment. Matings between sterile males and wild females yield infertile eggs, which reduce the reproductive potential of the wild population. The success of the SIT depends, to a large degree, on the ability of released, sterile males to attract and obtain matings with wild females. This consideration is especially important for species, such as *C. capitata*, in which females display a high degree of mate discrimination, based apparently on male courtship performance (Whittier et al., 1992, 1994).

Unfortunately, the mass-rearing procedures inherent to the SIT often lead to a reduction in the mating competitiveness of released *C. capitata* males. Owing to genetic drift and artificial selection in the mass-rearing environment (Leppla & Ozaki, 1991), sterile males typically have low mating success relative to wild males (Rossler, 1975; Shelly et al., 1994; McInnis et al., 1996; Lance et al., 2000). However, aside from replacing strains frequently or managing a

*Correspondence: USDA-APHIS, 41–650 Ahiki Street, Waimanalo, HI 96795 USA. E-mail: todd.e.shelly@aphis.usda.gov

colony under 'relaxed conditions' (Fisher & Caceres, 2000), there is no effective way to avoid this decrease in mating competitiveness, and for the recently developed genetic sexing strains (e.g., temperature sensitive lethal, *tsl*), such replacement requires considerable time and effort (Franz et al., 1996).

Thus, a persistent and important challenge for SIT is the development of simple and inexpensive means to enhance the mating performance of sterile *C. capitata* males released in the wild. One potentially productive approach involves the prerelease exposure of males to particular attractants. In particular, male exposure to ginger root oil [*Zingiber officinale* Roscoe (Zingiberaceae); hereafter (GRO)], which contains the known male attractant α-copaene (Flath et al., 1994a, b), has been shown to increase mating success dramatically in field cage trials (Shelly et al., 2004a and references therein; see Papadopoulos et al., 2001 and Shelly et al., 2004b for similar results with citrus peel oil). This result has been obtained for both wild and mass-reared males representing several different populations (strains) and tested in various pairwise combinations. These studies further showed that the oil's aroma alone was sufficient to boost male mating success and an increase in mating success was evident for as long as 8–10 days after exposure.

In a recent study, Shelly et al. (2004a) showed that pre-release exposure to GRO could be accomplished on a scale suitable for SIT programs. Specifically, several sterile release programs use plastic adult rearing containers (0.48 × 0.60 × 0.33 m; so-called PARC boxes, hereafter termed storage boxes) to hold mature pupae and newly emerged adults before release (≈ 36,000 flies per box). For a range of doses (0.0625–1.0 ml), the application of GRO to individual storage boxes resulted in a significant increase in the mating success of sterile males competing against wild males for copulations with wild females. For example, sterile males exposed to 1.0 ml of GRO for a 24-h period before testing obtained an average of 52% of the total matings per trial compared to only 24% for sterile males not exposed to the oil. Related tests further showed that exposure to GRO had no negative effect on male longevity (Shelly et al., 2004a) or flight endurance (J. Zermeno, unpubl.).

Although these studies suggest that exposure to GRO, a procedure dubbed 'aromatherapy' (Shelly et al., 2004a), might increase the effectiveness of SIT, previous assessment of chemical exposure has relied exclusively on mating tests conducted on individually caged host trees (or artificial trees, in some cases) and lasting only several hours. While far superior to laboratory trials conducted in small cages, short-term tests conducted on single trees obviously do not duplicate natural conditions as they preclude potential effects of spatial and temporal habitat heterogeneity and intertree movement on male mating competition. In addition, data on mating success, while valuable, provide only an indirect assessment of the potential benefit of aromatherapy, because they do not necessarily mirror the level of egg sterility realized. For example, short-term trials ignore the possibility that females may mate multiply, even on the same day (Vera et al., 2003). Given second male advantage in sperm competition in *C. capitata* (Saul & McCombs, 1993), mating with a GRO-exposed sterile male may not result in low hatch rate if the female remates with a wild male (Katiyar & Ramirez, 1970).

To evaluate more rigorously the effectiveness of GRO exposure, we here describe two projects that compared levels of egg sterility or pupal yield, respectively, following the release of wild flies and either GRO-exposed (treated) sterile males or GRO-deprived (control) sterile males in large field enclosures. As described in succeeding paragraphs, these enclosures contained multiple host plants and were much larger than the cages placed over single trees. While obviously not a complete substitute for open field tests, the large enclosures provided a more natural setting than the single-tree tents and, just as importantly, allowed for experimental manipulation of overflooding (sterile male : wild male) ratios and for replicated trials at the selected test ratios.

Materials and methods

The two projects were conducted in Hawaii and Guatemala, respectively. Because there were substantial differences in procedures, measurements, and analyses, the respective methods and results are presented separately for the two projects.

A. Hawaii

Study insects. Mass-reared flies were from a *tsl* strain (Vienna-7/Tol-99) produced by the California Department of Food and Agriculture Hawaii Fruit Fly Rearing Facility, Waimanalo, Oahu. Like other *tsl* strains, Vienna-7/Tol-99 possesses a sex-linked mutation, such that treating eggs with high temperature kills all female zygotes, thereby allowing production of males exclusively (Franz et al., 1996). Larvae of the mass-reared strain were reared on a standard diet (Tanaka et al., 1969). Males used in the current study were dyed and irradiated as pupae 2 days before eclosion under hypoxia at 150 Gy of gamma irradiation from a ^{137}Cs source. After irradiation, pupae were placed in paper bags, which, in turn, were placed inside the individual storage boxes. For the 5 : 1 and 10 : 1 overflooding ratios, 100 ml of pupae (1 ml ≈ 60 pupae) were placed in each of six paper bags (following the protocol of the California and Guatemala SIT programs),

while 63 ml of pupae were placed in each of two or four paper bags for the 30 : 1 and 60 : 1 overflooding ratios, respectively (release protocols for the different overflooding ratios are described below). Most adult emergence occurred 2 days after pupal placement, and emerging *tsl* males were fed a sugar-agar gel placed on the screened opening on top of the box. Storage boxes were maintained at 20–24 °C and 60–90% r.h. and received both natural and artificial light with a photoperiod of L12 : D12.

Because wild flies were not available in sufficiently larger numbers, we used 'wild-like' flies in the experiments. These flies derived from laboratory colonies reared from coffee berries (*Caffea arabica* L.) collected on Kauai. Adults were held in screen cages and provided with a sugar–protein (yeast hydrolysate) mixture (3 : 1 by weight), water, and an oviposition substrate (perforated plastic vials containing small sponges soaked in lemon juice). Eggs were placed on standard larval diet in plastic containers over vermiculite for pupation. Adults used in this study were separated by sex within 24 h of eclosion, well before reaching sexual maturity at 5–7 days of age (TE Shelly, unpubl.), and kept in screen-covered buckets (5 l volume 100–125 flies per bucket) with ample food (sugar–protein mixture) and water under the same conditions described above for mass-reared males. In all field tests, wild-like flies were 7–12 days old on the day of release. When used in the present study, the wild-like flies were 3–7 generations removed from the wild.

GRO exposure. The GRO was obtained from the Citrus and Allied Essences Ltd. (Lake Success, NY) and contained the hydrocarbon sesquiterpene α-copaene in low concentration (0.4%, TW Phillips, pers. comm.). The same exposure protocol was used in all experiments: GRO (1 ml) was applied to storage boxes for a 24-h period starting 3 days after the day of peak adult emergence. Using a pipette, we applied GRO to a 10-cm square of blotter paper. The oil-laden paper was then placed on the screened opening on top of the storage box (not touching the food gel), because Shelly (2001) found that exposure to the oil's aroma alone (i.e., without direct contact with the oil) conferred a mating advantage (in fact, when given access to GRO, males do not feed on it but instead become quiescent). An empty storage box was then placed on the treated box to mimic SIT programs, where storage boxes are stacked to save space. For each storage box set up with GRO, we also set up a storage box that received the same quantity of pupae but no GRO, thus yielding non-exposed (control) tsl males. In all instances, boxes receiving GRO were kept in a separate room from those not receiving the treatment to prevent inadvertent exposure of control *tsl* males. In all field tests, both treated and control *tsl* males were released at 5 days of age, i.e., 1 day after the removal of the GRO from the treated storage boxes. Wild-like males were not exposed to GRO in any experiment.

Field protocol. Trials were conducted in two nylon-screen enclosures (16 m long × 6 m wide × 2.5 m high) set up in a guava orchard (*Psidium guajava* L.) in Waimanalo, Oahu (elevation 20 m). The tents, which were parallel to one another and separated by 5 m, contained 10 and 12 guava trees, respectively, and were covered with shade screen to reduce insolation. Treated and control *tsl* males were tested concurrently (i.e., one treatment per enclosure), and treatments were alternated between the two enclosures in successive replicates. Trials were conducted during February–July in 2003 and 2004. Daily maximum and minimum air temperatures were recorded over the entire study at a weather station situated ca. 100 m from the enclosures.

We measured levels of egg sterility under four overflooding ratios: 5 : 1, 10 : 1, 30 : 1, and 60 : 1, respectively. Two hundred wild-like males and 200 wild-like females were released for all ratios except 10 : 1, where 100 wild-like individuals of each sex were released. Mass-reared males were counted individually for the 5 : 1 and 10 : 1 ratios. In these cases, we removed one paper bag (and the males resting on it) from the storage box, quickly transferred it to a screen cage (30-cm cube), removed the appropriate number of males using an aspirator, and placed them in a plastic bucket for transportation to the field. Based on quality control data (California Department of Agriculture, unpubl.), we estimated that 10% of eclosed *tsl* males were incapable of flight. To compensate for these individuals, we counted and released 1111 males (≈1000 flight-capable males) for the 5 : 1 (1000/200) and the 10 : 1 (1000/100) overflooding ratios, respectively. For the 30 : 1 and 60 : 1 ratios, males were released directly from the storage boxes into the field enclosure. The aforementioned pupal volumes were chosen to compensate for the fact that only about 80% of *tsl* male pupae yield adults capable of flight (California Department of Agriculture, unpubl.). For the 30 : 1 overflooding ratio, the storage boxes held 126 ml of pupae (63 ml in each of two paper bags) or an estimated 6048 flight-capable, *tsl* males (126 ml pupae × 60 pupae/ml × 0.80 fliers; 6048/200 ≈ 30 : 1 overflooding ratio). The same computations apply to the 60 : 1 overflooding ratio and the placement of 252 ml of pupae in individual storage boxes (yielding ≈ 12,096 flight-capable *tsl* males; 12,096/200 ≈ 60 : 1 ratio). In no instance were the *tsl* males exposed to a period of prerelease cooling, a procedure adopted for aerial releases. However, earlier data (Shelly et al., 2004a) showed that such cooling had no effect on the mating performance of GRO-exposed males.

The same schedule was followed for all trials. On day 1, food and water were introduced, and flies were released.

Food (sugar–protein mixture) was presented in Petri dishes held within Jackson traps (lacking sticky inserts) suspended 1.5–2.25 m above ground from tree branches at four evenly spaced locations. At each of these sites, we also provided water in a covered plastic cup (100 ml vol with an emerging cotton wick) held within a Jackson trap. The wires suspending the resource-laden Jackson traps from branches were coated with Tanglefoot® (Tanglefoot Company, Grand Rapids, MI, USA) to exclude ants. Food and water sources were not replaced during a trial. Following the placement of food and water, males were released 20 min before females by placing the plastic buckets or the storage box in the center of an enclosure, removing the cover, and allowing males to fly away. The containers were tapped periodically to induce male flight, and approximately 5 min before female release, the containers were inverted and tapped firmly to remove any remaining males. The male containers were then removed from the tent, and females were released from the center of the tent. Flies were released between 09:00 and 10:00 hours for all trials.

On day 2, 12 Granny Smith apples (*Malus domestica* Borkh.) were placed in the enclosures at 10:00 hours for oviposition. Apples were suspended 1.5–2.5 m above ground by piercing the fruit with a nail and connecting the nail to a branch with wire. Tanglefoot® was applied to the wire to exclude ants. The apples served as the only available oviposition resource as guava fruits were removed before the trials. On days 3 and 4 at 10:00 hours, apples were collected and replaced with new ones. On day 5, apples were collected but not replaced, marking the end of the trial (food and water were also removed at this time). Hereafter, the apples placed in the enclosures on days 2, 3, and 4 are referred to collectively as batches 1, 2, and 3, respectively.

For each trial, we also measured egg hatch of wild females mated exclusively to wild males in a field-cage over a single guava tree adjacent to the large enclosures. Two hundred individuals of each sex were introduced on day 1, and two apples were introduced on day 2 for a 24-h period.

Upon collection, apples were returned to the laboratory, examined for oviposition 'sting' marks under a dissecting microscope, and eggs were removed using a scalpel and fine forceps. Eggs were placed on moistened blotter paper within Petri dishes and then incubated at 27 °C for 48 h. Hatch was then determined by re-examining the eggs under a dissecting microscope.

Seven replicates were conducted for each overflooding ratio, where a replicate consisted of treated and control *tsl* males placed concurrently, but separately, in the large enclosures (with associated wild-like flies) and wild-like flies exclusively placed in the single, field-caged guava tree. Successive replicates were usually separated by 7 days. The minimum interval between replicates was 3 days, and in these instances two sticky traps, one baited with trimedlure (male attractant) and the other with honey–yeast mixture, were placed in each enclosure after apple collection on the final day of a replicate and left in place until the next release. In addition, we searched each enclosure for about 1 h just before the next release and captured any surviving flies.

Fried's Competitiveness (C) Index. For each replicate, we computed Fried (1971) competitiveness index (C) to compare the performance of control and treated tsl males vs. wild-like males, where $C = (W/S) \times [(H_w - H_c)/(H_c - H_s)]$, with W = number of wild-like males released in the test enclosure, S = number of sterile (tsl) males released in the test enclosure, H_w = percentage of egg hatch from wild-like females following mating with wild-like males exclusively (as determined from the single, caged guava tree), H_c = percentage of egg hatch from wild-like females in the test enclosure, and H_s = percentage of egg hatch from wild-like females following mating with sterile (tsl) males. To measure H_s, we mated wild-like females (7–13 days old) with sterile *tsl* males (5–6 days old) in the laboratory and then placed the females singly in 1-l containers with food and water. On the following day, oviposition vials (as described previously) were placed in the containers, and eggs were collected and counted 24 h later. Egg hatch was monitored following the protocol described previously. Only females that laid a minimum of 10 eggs were included in the analysis. Based on these measurements, $H_s = 0.003$ (2/728; n = 45 females).

Clutch size and daily egg output. To better interpret the data collected on egg abundance among individual oviposition holes, we made observations in the laboratory to estimate the number of eggs laid per oviposition bout (i.e., clutch size) and the total number of eggs laid per day. To estimate clutch size, we placed individual, wild-like females (8–12 days old, mated 1 day earlier to wild-like males) in screen cages (30-cm cubes) and 30 min later introduced a single apple in which we made eight to 10 shallow holes with a dissecting needle. Holes were made to facilitate egg-laying, and all females that oviposited did so in these premade holes. Females were placed in the cages between 09:00 and 09:30 hours and observed continuously until oviposition or 1 h had elapsed. Once oviposition ceased, the apple was removed, and eggs in the appropriate hole were counted.

To estimate daily fecundity, we placed individual females in screen cages along with a single apple containing 8–10 holes as described above. Females were placed in the cages at 08:00 hours and removed at 16:00 hours. Apples were kept in a refrigerator (12–15 °C) overnight, and total egg counts were made the following morning. Because trials did not span the entire dawn–dusk period

(approximately 12 h), our estimates of daily fecundity are likely conservative.

Statistical analyses. Because, in most instances, the data met assumptions of normality and equal variance, intergroup comparisons were made using ANOVA. Data on total egg abundance were analyzed using two-way ANOVA with overflooding ratio and male treatment category (GRO-exposed or GRO-deprived) as the factors. Egg numbers were compared on a per female basis to accommodate the lower number of females used at the 10 : 1 ratio (100 females rather than 200 females used for all other ratios), and values were $\log_{10}$ transformed (using x + 1) to normalize the data. To investigate temporal patterns of egg-laying, egg numbers from successive batches of apples were converted to proportions of the total egg output for a given replicate. These values were then arcsine transformed and used in a three-way ANOVA with overflooding ratio, male treatment category, and apple batch number as the factors. Levels of egg sterility (number of unhatched eggs/total eggs) were analyzed (using arcsine transformed values) using a two-way ANOVA with overflooding ratio and male treatment category as the factors. When significant variation was detected overall, the Tukey multiple comparisons test was used to identify pairwise differences ($P = 0.05$ in all tests). Mean values ± 1 SE are presented. Where the parametric assumptions were not met, the Kruskal–Wallis (ANOVA on ranks, test statistic H) and Mann–Whitney (two-sample location on ranks, test statistic T) tests were performed.

Although the daily maximum temperatures varied significantly among the time intervals when the four overflooding ratios were investigated ($H = 109.6$, d.f. = 3, $P<0.001$), we excluded temperature as a factor in the analyses for the following reasons. First, the level of variation, while consistent, was quite small. Among the study periods, the mean maximum temperatures ranged only between 25.6–29.5 °C in 2003 and 25.8–30.0 °C in 2004. Second, and most likely reflecting this low variation, we found no correlation between the mean daily maximum temperature during a replicate and (1) the total number of eggs collected, (2) the total number of apples containing eggs, (3) the total number of oviposition sites (holes), or (4) the proportion of sterile eggs when considering data from the GRO treatments (control or treated) separately, or collectively, within or between overflooding ratios ($P>0.05$ in all cases; Spearman rank correlation; analyses included the 5 : 1, 30 : 1, and the 60 : 1 ratios among which the same number of wild-like flies was released per replicate).

B. Guatemala

For the sake of brevity, we focus on describing procedures that were unique to Guatemala, and unless otherwise stated, the protocol described previously for Hawaii was followed in Guatemala as well.

Study insects. Mass-reared flies were from the same *tsl* strain used in Hawaii and were obtained from the Moscamed Program facility in El Pino, Guatemala. Males used in this study were dyed and irradiated as pupae 2 days before eclosion in air at 100 Gy of gamma radiation from a ^{60}Co source. In Guatemala, we used only a single overflooding ratio, namely 25 : 1, with the same number of wild flies and tsl males used over all replicates. As 100 wild males (and 100 wild females) were used per replicate, we placed 52 ml of pupae (where 1 ml ≈ 69 pupae for a total of ≈ 3588 pupae) per paper bag with each storage box (same type as used in Hawaii) receiving a single paper bag. Quality control data for the El Pino facility showed that only 70% of tsl pupae produce flying males (P Rendon, unpubl.). Therefore, approximately 2512 flight-capable, tsl males (3588 pupae × 0.70 fliers) were released from individual storage boxes (2512/100 ≈ 25 : 1 overflooding ratio).

Wild flies were reared from coffee berries collected near Antigua. Berries were placed over vermiculite, which was sifted daily for pupae. Adults were separated within 24 h of eclosion, well before reaching sexual maturity at 8–10 days of age (P Rendon, unpubl.) and kept in screen-covered plastic cups (400 ml; 50 flies per cup) with ample food (sugar–protein mixture) and water. In all field tests, wild flies were 9 days old when released.

GRO exposure. The protocol for GRO exposure was the same as in Hawaii, except that in Guatemala we used a dose of 0.5 ml of GRO per storage box.

Field protocol. Trials were conducted in March 2003, using 15 nylon-screen enclosures (same dimensions as those used in Hawaii) set up in a coffee plantation near Antigua (elevation 1800 m). During the study period, maximum daily temperatures ranged from 26 to 30 °C. A nylon curtain was sewn inside each tent at mid-length, resulting in two compartments per enclosure (each 8 m long × 6 m wide × 2.5 m high), i.e., 30 compartments in total. Each compartment contained 12 coffee plants. The compartments were assigned randomly to one of the following treatments (10 compartments per treatment): wild flies only, wild flies plus GRO-exposed *tsl* males, or wild flies plus GRO-deprived *tsl* males. As noted above, a constant number of wild flies (100 per sex) and *tsl* males (52 ml or ≈ 2512 flight-capable *tsl* males) was released per compartment.

The chief difference between the Hawaiian and Guatemala projects involved the method used for assessing the competitive ability of GRO-exposed vs. GRO-deprived *tsl*

Table 1 Daily and total number of *Ceratitis capitata* eggs collected from apples under the four overflooding ratios tested in Hawaii. In all cases, with (GRO) and without (CON) exposure to ginger root oil, 200 wild-like individuals of each sex were released in the enclosures, except for the 10 : 1 ratio where 100 wild-like individuals of each sex were released (ANOVA on egg output was performed on a per female basis as described in the text). Flies were released on day 1; apples in each batch were placed in the enclosure for 24 h, starting with batch 1, which was introduced on day 2. Mean values (±1 SE) are presented (rounded to nearest integer); seven replicates were performed for all ratios

	Number of eggs					
Overflooding ratio	Batch 1	Batch 2	Batch 3	Total	t	P
5 : 1 CON	370 (103)	159 (65)	32 (10)	561 (165)	0.30	0.80
5 : 1 GRO	354 (89)	100 (25)	51 (24)	505 (134)		
10 : 1 CON	139 (25)	61 (26)	30 (12)	230 (53)	1.02	0.33
10 : 1 GRO	100 (18)	46 (6)	23 (6)	169 (23)		
30 : 1 CON	379 (94)	79 (22)	30 (6)	488 (105)	0.07	0.95
30 : 1 GRO	309 (77)	91 (30)	77 (32)	477 (117)		
60 : 1 CON	770 (114)	128 (33)	144 (39)	1042 (161)	0.52	0.61
60 : 1 GRO	797 (169)	201 (51)	230 (132)	1228 (317)		

males. Whereas we measured egg sterility directly in Hawaii, in Guatemala we recorded the number of pupae produced from mango fruits (*Mangifera indica* L.) placed in the compartments for oviposition (coffee berries were removed prior to the experiment). On the day flies were released, we hung one ripe mango (1.5–2 m above ground) on each of 12 coffee plants distributed evenly in a compartment. One week later, we removed the mangoes and placed them over sawdust in the laboratory at 25–28 °C. Pupae were then sifted and counted 2 weeks later.

Statistical analyses. Pupal yield was compared among treatments with a one-way ANOVA, followed with the Tukey multiple comparisons test (P = 0.05 in all tests).

Results

A. Hawaii

Total and daily egg production. Raw data on total and daily egg collections are presented in Table 1. The total number of eggs collected per female showed significant variation with overflooding ratio ($F_{3,48} = 9.29$, $P<0.001$) but not with GRO treatment category ($F_{1,48} = 0.10$, $P = 0.75$). The interaction between overflooding ratio and GRO treatment was not significant ($F_{3,48} = 0.16$, $P = 0.92$). The subsequent Tukey test revealed that egg number per female was significantly higher at the 60 : 1 ratio than at the remaining three overflooding ratios, among which there was no significant variation.

Independent of the overflooding ratio or male treatment status, most of the eggs collected during a given trial were from the initial batch of apples, with egg numbers declining between successive batches (Table 1; Figure 1). Results

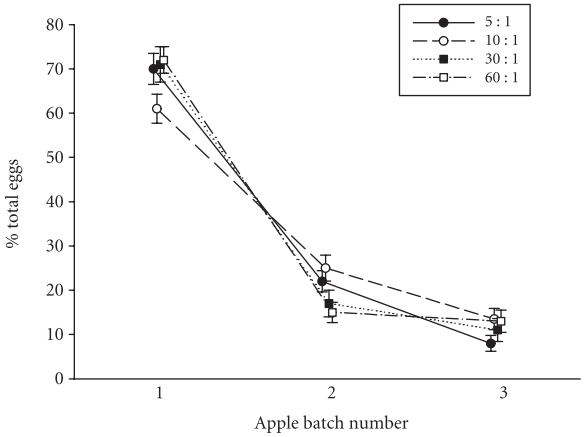

Figure 1 Relative number of *Ceratitis capitata* eggs collected (% total) from successive batches of apples for the four overflooding ratios tested in Hawaii. Symbols represent mean values (±1 SE; n = 7). Flies were released on day 1; apples in each batch were placed in the enclosure for 24 h, starting with batch 1, which was introduced on day 2. For a given ratio, data from control and treated *tsl* males were combined for a given batch of apples as ANOVA revealed no effect of male treatment category on the level or timing of egg production.

of the three-way ANOVA showed that the relative number of eggs collected from different apples batches did not vary significantly with overflooding ratio ($F_{3,144} = 0.04$, $P = 0.99$) or GRO treatment category ($F_{1,144} = 0.05$, $P = 0.82$) but varied significantly with apple batch number ($F_{2,144} = 328.5$, $P<0.001$). None of the interaction terms were significant ($P>0.05$ in all cases). Over all replicates (n = 56), the first batch of apples contained an average of 68.2 ± 1.92% of the total egg count, with values ranging from 43 to 96%. In contrast, the second and third batches of apples contained,

on average, 19.9 ± 1.4% (range: 1–43%) and 11.3 ± 1.2% (range: 0–35%) of the total egg production, respectively. The multiple comparisons test revealed significant differences between all three batches (i.e., batch 1 > batch 2 > batch 3; $P<0.001$ in all cases).

Egg distribution among holes and apples. Although ovipositional behavior was not the focus of our study, field data on egg distribution, coupled with our laboratory observations, confirmed an earlier study (Papaj et al., 1989) and showed that females preferred to oviposit in existing holes rather than boring new ones. In dissecting apples from the field enclosures, we generally found eggs in a relatively small number of holes over all available apples. To illustrate this point, we use data from the first batch of apples over all replicates ($n = 28$) for the 5 : 1 and 30 : 1 overflooding ratios, which had an equal number of wild-like flies released and similar total egg production. These data revealed that, on average, 345.9 ± 43.9 eggs (range: 75–834) were distributed among 10.8 ± 1.1 holes (range: 2–24) occurring on 6.1 ± 0.3 (range: 3–9) different apples. The mean number of eggs/hole (34.7 ± 3.3; range: 9.5–86.5) was approximately seven times larger than the mean clutch size observed in the laboratory (5.2 ± 0.5; range: 1–15; $n = 43$), and in 15 instances we observed more than 100 eggs in a single hole.

Daily fecundity in the laboratory. Egg production of 61 newly mated females was monitored for an 8-h period on the first day available for oviposition. Females laid, on average, 14.3 ± 1.8 eggs, with values ranging from zero ($n = 17$ females) to 56 eggs. Assuming an output of 14 eggs per female on day 2 of the trials (apple batch 1, which provided the first opportunity for egg-laying), we estimate (based on mean egg collections for batch 1; Table 1) that 22–27 females deposited eggs in apple batch 1 for the overflooding ratios of 5 : 1 and 30 : 1, 7–10 females laid eggs in apple batch 1 for the 10 : 1 overflooding ratio, and 55 females laid eggs in apple batch 1 for the 60 : 1 overflooding ratio.

Egg sterility and competitiveness (C) values. Levels of egg sterility varied significantly with overflooding ratio ($F_{3,48} = 8.4$, $P<0.001$) and male treatment category ($F_{1,48} = 30.2$; Figure 2). The interaction term was not significant ($F_{3,48} = 0.9$, $P = 0.47$). Closer examination of the data revealed that the effect of male treatment was significant at all overflooding ratios (t-tests: $P<0.05$ in all cases), with egg sterility levels being, on average, 7–28% higher for treated *tsl* males than control *tsl* males. However, the degree to which egg sterility varied with overflooding ratio differed between the control and treated *tsl* males. Egg sterility levels for treated *tsl* males were fairly consistent across

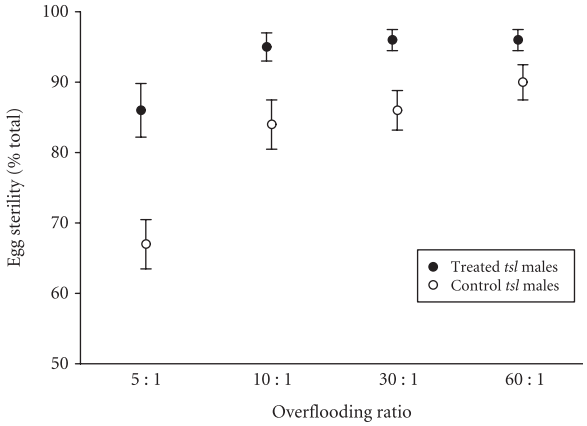

Figure 2 Relative number of sterile (unhatched) *Ceratitis capitata* eggs collected (% total) for treated and control *tsl* males at the four overflooding ratios tested in Hawaii. Symbols represent mean values (± 1 SE; $n = 7$).

different overflooding ratios (range: 86%–96%; Figure 2), and the between-ratio variation in sterility level was not significant for these males ($H = 3.0$, d.f. = 3, $P = 0.39$). In contrast, average sterility levels associated with control *tsl* males showed greater variation (Figure 2), increasing from an average of 67% at the 5 : 1 overflooding ratio to 89% for the 60 : 1 overflooding ratio (Figure 2). Correspondingly, sterility levels varied significantly with overflooding ratio for control *tsl* males ($F_{3,24} = 7.1$, $P = 0.002$). Interestingly, from an applied perspective, the percent sterility observed for treated *tsl* males at the 5 : 1 ratio was similar to that observed for control *tsl* males at all higher overflooding ratios ($F_{3,24} = 0.4$, $P = 0.75$), including even 60 : 1.

Sterility did not appear to vary substantially among the different batches of apples. For treated *tsl* males, we compared sterility levels for eggs collected from apple batches 1, 2, and 3, respectively, using pooled data over all overflooding ratios (given the absence of significant variation in overall sterility among the different ratios, see above). This analysis failed to detect significant between-batch variation in sterility level ($H = 2.8$, d.f. = 2, $P = 0.24$). For control *tsl* males, we examined between-batch variation in sterility separately for each overflooding ratio given the significant variation in overall sterility among overflooding ratios noted above. For all ratios, we found that sterility level varied independently of batch number ($F_{2,18}$ range = 0.49–2.86, $P>0.05$ in all cases).

Values of Fried's Competitiveness Index (C) reflected the relationships described above between overflooding ratio and egg sterility (Figure 3). For treated *tsl* males, the relatively constant level of egg sterility recorded over different overflooding ratios resulted in decreasing competitiveness values with increasing overflooding ratio ($H = 9.3$,

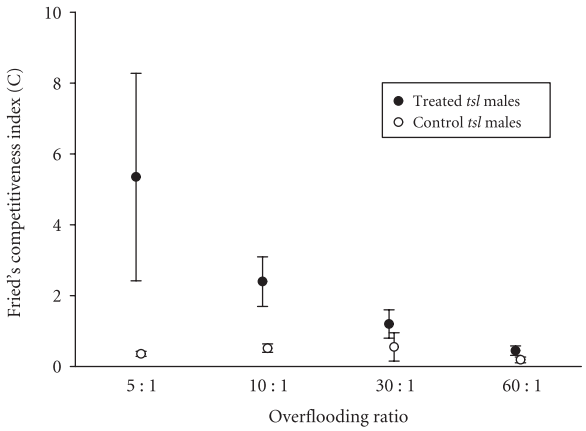

Figure 3 Fried's Competitiveness Index (C) for treated and control *Ceratitis capitata tsl* males at the four overflooding ratios tested in Hawaii. Symbols represent mean values (±1 SE; n = 7).

d.f. = 3, P = 0.02). In contrast, because egg sterility increased with increasing overflooding ratio, C-values for control *tsl* males remained more or less constant (H = 7.75, d.f. = 3, P = 0.07). Despite the convergence in C-values with increasing overflooding ratio, these values were significantly greater for treated *tsl* males than control *tsl* males at each overflooding ratio tested (Mann–Whitney test: P<0.05 in all cases; $n_1 = n_2 = 7$). In computing C, we found that H_w averaged 78.2% (±1.9; range: 66–92%) over all replicates (n = 28) and that H_w did not vary significantly among the overflooding ratios ($F_{3,24} = 1.4$, P = 0.28).

B. Guatemala

The average numbers of pupae collected in the three treatments were 1399 ± 430 (range: 653–2083) for wild flies only, 223 ± 126 (107–307) for wild flies plus GRO-exposed *tsl* males, and 291 ± 86 (151–589) for wild flies plus GRO-deprived *tsl* males (n = 10 compartments/treatment). There was significant variation in the number of pupae collected among the treatments ($F_{2,27} = 63.2$, P<0.001). The number of pupae collected in the wild fly only treatment was significantly greater than that recorded for the other two treatments (Tukey test). No difference was detected between the treatments involving the release of GRO-exposed or GRO-deprived *tsl* males (Tukey test), even though pupal yield was, on average, 23% lower (223/291) for compartments with GRO-exposed *tsl* males than compartments with non-exposed *tsl* males.

Discussion

Our studies in Hawaii and Guatemala generated different results regarding the effectiveness of GRO exposure in enhancing the mating competitiveness of sterile males of *C. capitata*. In Hawaii, exposing *tsl* males to GRO enhanced their mating competitiveness relative to wild-like males and correspondingly increased the proportion of sterile eggs in stung fruits. This GRO-mediated advantage was evident at overflooding ratios ranging from 5 : 1 to 60 : 1, as both the C-values and levels of egg sterility associated with treated *tsl* males were significantly greater than those of control *tsl* males for all overflooding ratios tested. In Guatemala, by contrast, at the single overflooding ratio tested (25 : 1), we found no significant difference in the total number of pupae collected from infested fruits in compartments containing wild flies and either GRO-exposed or GRO-deprived *tsl* males, indicating no effect of GRO exposure on the mating competitiveness of the *tsl* males.

The different outcomes may have been a direct result of the different protocols used at the two sites. In particular, we suspect that pupal counts, as used in Guatemala, were not sufficiently sensitive to detect an effect of GRO exposure. Because pupal yield was an absolute, and not a relative, measure, detection of a treatment effect required low variability in the number of eggs laid (and the number of ovipositing females) and/or larval mortality among compartments receiving *tsl* males. Otherwise, variation in pupal yield might reflect variation in oviposition and/or survivorship independent of male GRO treatment. However, data from the compartments receiving wild flies only (where GRO treatment was not a confounding factor) show large variation in pupal yield. Despite similar conditions, pupal counts varied over 300% (2083/653 = 3.19) among the 10 compartments containing only wild flies. With such large, inherent variability, detecting a relatively small difference between treatment means was difficult given the relatively small sample sizes used (10 replicates per treatment).

Although the data from Hawaii indicated that prerelease, GRO exposure might increase the effectiveness of medfly SIT, several factors potentially confound this interpretation. First, as some control programs release sterile males on a weekly basis, our tests (lasting only 5 days) may not have detected changes in male mating competitiveness possibly occurring over slightly longer intervals. Thus, the possibility exists that GRO-exposed males have high mating competitiveness within a few days of release but show declining competitiveness thereafter. Such a decline could result from several, non-mutually exclusive, factors: diminished effect ('wearing off') of GRO exposure, high mortality (relative to control *tsl* males), or female remating with wild males. While these factors can not be discounted, data from earlier studies suggest that their impact is small: GRO-exposure was shown to confer a mating advantage for over a week (Shelly, 2001), field trials revealed no negative effect of GRO exposure on survivorship of *tsl* males in

field cages (Shelly et al., 2004a), and females mated initially to GRO-exposed *tsl* males had a lower, not higher, propensity to remate with wild males than females first mated to control *tsl* males (Shelly et al., 2004c).

In addition, the use of enclosures as an experimental tool could also compromise interpretation of the present findings, because enclosures preclude dispersal as an influence on male mating competitiveness. If GRO exposure reduces flight propensity or ability, aromatically treated *tsl* males may be less likely to locate preferred habitat, including active mating (lek) sites, and hence display decreased mating competitiveness. However, two sets of data indicate that GRO exposure has no negative impact on male dispersal. Using males tethered to a flight mill (in which males were free to fly in a circular path), J Zermeno (unpubl.) found that flight duration and speed did not differ significantly between GRO-exposed and control *tsl* males. In addition, following aerial release in Florida, similar numbers of GRO-exposed and GRO-deprived *tsl* males were captured in trimedlure-baited traps suspended in host trees (TE Shelly, unpubl.). This latter study also showed that the post-release interval over which males were trapped was actually greater for GRO-exposed than GRO-deprived males (mean values = 17 and 11 days, respectively; n = 7 releases; TE Shelly, unpubl.), indicating greater field longevity for GRO-exposed *tsl* males.

Use of field enclosures did appear to generate higher levels of egg sterility than typically observed under field conditions. In the large enclosures, we found that egg sterility achieved by control *tsl* males was 80–90% for overflooding ratios ranging from 10 : 1 to 60 : 1. In contrast, Wong et al. (1986), working in mixed fruit orchards on Maui, Hawaii, observed a similar level of egg sterility only at much higher overflooding ratios, i.e., 100 : 1–400 : 1. Even more dramatically, in a study of *C. capitata* in Guatemalan coffee fields, Rendon et al. (2004) found that only 30% of the collected eggs were sterile at an overflooding ratio of 500 : 1 and the maximum sterility level (75%) was achieved only when the overflooding ratio exceeded 1300 : 1. The higher levels of egg sterility noted for control *tsl* males in the present study most likely derived from the fact that the males were not required to disperse (and locate lek sites), feed, or avoid predators prior to encountering sexually receptive females. In the field, high mortality incurred even before encountering a potential mate was most likely responsible for the reduced effectiveness of sterile male releases.

Although our experimental protocol obviously did not exactly mimic the real world, preliminary data from Hawaiian coffee fields also indicate that GRO exposure increases the mating competitiveness of *tsl* males. Over approximately 2.5 months in 2003, we made weekly releases of GRO-exposed *tsl* males in one plot and non-exposed *tsl* males in another, nearby plot. Weekly estimates (n = 12) of mating competitiveness, based on egg sterility levels, were consistently higher for GRO-exposed than for non-exposed *tsl* males (mean values: 0.54 vs. 0.14, respectively). We are currently repeating this experiment (alternating the plots receiving treated or control tsl males) and will report the findings in a future article.

In conclusion, the results described above for control *tsl* males in the Hawaiian study appear to characterize the accepted modus operandi of medfly SIT programs, i.e., the competitive inferiority of sterile males necessitates the release of a very large number of males, which numerically swamp the wild population. In other words, medfly management now appears to depend primarily on the quantity of sterile males released and only secondarily on their quality. However, the present findings suggest that, above moderate overflooding ratios, any further increases in overflooding may generate only small increases in egg sterility. For control *tsl* males in Hawaii, egg sterility averaged 84% at a 10 : 1 overflooding ratio and increased to only 90% at a 60 : 1 overflooding ratio (Figure 2). Based on this trend, we would anticipate a very gradual, asymptotic rise to complete egg sterility with increasing overflooding ratio of control *tsl* males. As indicated by the Hawaiian study, the value of prerelease, GRO exposure derives from (1) reaching an asymptote at a lower overflooding ratio (an especially important concern where financial or logistic constraints limit the quantity of sterile males released) and (2) reaching a higher asymptote (Figure 2). Because of these two factors, it appears that complete sterility (leading to eradication) would be realized at lower overflooding ratios for GRO-exposed males. This conclusion echoes that of Barry et al. (2003), who, in a series of mating trials on field-caged host trees, found that GRO-exposed, sterile medfly males released in a 1 : 1 ratio with wild males obtained approximately the same proportion of total matings with wild females as non-exposed, sterile males released at an overflooding ratio of 10 : 1.

Acknowledgements

We are grateful to Roger Corrales for permission to use the Waimanalo facility and for the weather data and Joe Zermeno for permission to cite unpubl. For field and laboratory assistance, we thank Luc Le Blanc, Ron Lim, James Edu, Elaine Pahio, and Mindy Teruya in Hawaii and F. Acajabon, Carlos Dimas, Felipe Jeronimo, Pablo Matute, and Rolando Santos in Guatemala. TES would also like to thank Aracely Galan for administrative assistance in Guatemala. This research was supported in part by funds from the Binational Agricultural Research and Development Fund (BARD project no. US-32356–01) to B. Yuval and T.E.S.

References

Barry JD, Shelly TE, McInnis DO & Morse JG (2003) Potential for reducing overflooding ratios of sterile Mediterranean fruit flies (Diptera: Tephritidae) with the use of ginger root oil. Florida Entomologist 86: 29–33.

Fisher K & Caceres C (2000) A filter rearing system for mass reared genetic sexing strains of Mediterranean fruit fly (Diptera: Tephritidae). Area-wide Control of Fruit Flies and Other Insect Pests (ed. by K-H Tan), pp. 543–550. Penerbit Universiti Sains Malaysia, Pulau Pinang, Malaysia.

Flath RA, Cunningham RT, Mon TR & John JO (1994a) Additional male Mediterranean fruit fly (*Ceratitis capitata* Wied.) attractants from angelica seed oil (*Angelica archangelica* L.). Journal of Chemical Ecology 20: 1969–1984.

Flath RA, Cunningham RT, Mon TR & John JO (1994b) Male lures for Mediterranean fruit fly (*Ceratitis capitata* Wied.): structural analogues of α-copaene. Journal of Chemical Ecology 20: 2595–2609.

Franz G, Kerremans P, Rendon P & Hendrichs J (1996) Development and application of genetic sexing systems for the Mediterranean fruit fly based on a temperature sensitive lethal. Fruit Fly Pests: A World Assessment of Their Biology and Management (ed. by BA McPheron & GJ Steck), pp. 185–191. St. Lucie Press, Delray Beach, FL.

Fried M (1971) Determination of sterile-insect competitiveness. Journal of Economic Entomology 64: 869–872.

Hendrichs J, Franz G & Rendon P (1995) Increased effectiveness and applicability of the sterile insect technique through male-only releases for control of Mediterranean fruit flies during fruiting seasons. Journal of Applied Entomology 119: 371–377.

Hendrichs J, Robinson AS, Cayol JP & Enkerlin W (2002) Medfly areawide sterile insect technique programmes for prevention, suppression or eradication: the importance of mating behavior studies. Florida Entomologist 85: 1–13.

Katiyar KP & Ramirez E (1970) Mating frequency and fertility of Mediterranean fruit fly females alternately mated with normal and irradiated males. Journal of Economic Entomology 63: 1247–1250.

Lance DR, McInnis DO, Rendon P & Jackson CG (2000) Courtship among sterile and wild *Ceratitis capitata* (Diptera: Tephritidae) in field cages in Hawaii and Guatemala. Annals of the Entomological Society of America 93: 1179–1185.

Leppla N & Ozaki E (1991) Introduction of a wild strain and mass rearing of medfly. The International Symposium on the Biology and Control of Fruit Flies (ed. by K Kawasaki, O Iwahashi & KY Kaneshiro), pp. 148–154. University of the Ryukyus, Nishihara, Okinawa, Japan.

McInnis DO, Lance DR & Jackson CG (1996) Behavioral resistance to the sterile insect technique by Mediterranean fruit flies (Diptera: Tephritidae) in Hawaii. Annals of the Entomological Society of America 89: 739–744.

Papadopoulos NT, Katsoyannos BI, Kouloussis NA & Hendrichs J (2001) Effect of orange peel substances on mating competitiveness of male *Ceratitis capitata*. Entomologia Experimentalis et Applicata 99: 253–261.

Papaj DR, Katsoyannos BI & Hendrichs J (1989) Use of fruit wounds in oviposition by Mediterranean fruit flies. Entomologia Experimentalis et Applicata 53: 203–209.

Rendon P, McInnis DO, Lance D & Stewart J (2004) Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. Journal of Economic Entomology 97: 1547–1553.

Rossler Y (1975) The ability to inseminate: a comparison between laboratory-reared and field populations of the Mediterranean fruit fly (*Ceratitis capitata*). Entomologia Applicata et Experimentalis 18: 255–260.

Saul SH & McCombs SD (1993) Dynamics of sperm use in the Mediterranean fruit fly (Diptera: Tephritidae): reproductive fitness of multiple-mated females and sequentially mated males. Annals of the Entomological Society of America 86: 198–202.

Shelly TE (2001) Exposure to α-copaene and α-copaene-containing oils enhances mating success of male Mediterranean fruit flies (Diptera: Tephritidae). Annals of the Entomological Society of America 94: 497–502.

Shelly TE, Dang C & Kennelly (2004b) Exposure to orange (*Citrus sinensis* L.) trees, fruits, and oil enhances mating success of male Mediterranean fruit flies *Ceratitis capitata* (Wiedemann). Journal of Insect Behavior 17: 303–315.

Shelly TE, Edu J & Pahio E (2004c) Sterile males of the Mediterranean fruit fly exposed to ginger root oil induce female remating: implications for the sterile insect technique (Diptera: Tephritidae). Florida Entomologist 87: 628–629.

Shelly TE, McInnis DO, Pahio E & Edu J (2004a) Aromatherapy in the Mediterranean fruit fly (Diptera: Tephritidae): sterile males exposed to ginger root oil in prerelease storage boxes display increased mating competitiveness in field-cage trials. Journal of Economic Entomology 97: 846–853.

Shelly TE, Whittier TS & Kaneshiro KY (1994) Sterile insect release and the natural mating system of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). Annals of the Entomological Society of America 87: 470–481.

Tanaka N, Steiner LF, Ohinata K & Okamoto R (1969) Low-cost larval rearing medium for mass-production of oriental and Mediterranean fruit flies. Journal of Economic Entomology 62: 967–968.

Vera MT, Cladera JL, Calcagno G, Vilardi JC, McInnis DO & Field Working Group (2003) Remating of wild *Ceratitis capitata* (Diptera: Tephritidae) females in field cages. Annals of the Entomological Society of America 96: 563–570.

Whittier TS, Kaneshiro KY & Prescott LD (1992) Mating behavior of Mediterranean fruit flies (Diptera: Tephritidae) in a natural environment. Annals of the Entomological Society of America 85: 214–218.

Whittier TS, Nam FY, Shelly TE & Kaneshiro KY (1994) Male courtship success and female discrimination in the Mediterranean fruit fly (Diptera: Tephritidae). Journal of Insect Behavior 7: 159–170.

Wong TTY, Kobayashi RM & McInnis DO (1986) Mediterranean fruit fly (Diptera: Tephritidae): methods of assessing the effectiveness of sterile insect releases. Journal of Economic Entomology 79: 1501–1506.